ATTACHMENT III - PROTOCOL

Ecolab
Study Identification Number 1800076

REGULATED PESTICIDE EFFICACY STUDY PROTOCOL

STUDY TITLE: CW32A Supplemental Food Contact Sanitizing Efficacy

EPA REG. NO.: 1677-[pending]

ECOLAB GLP STUDY NUMBER: 1800076

PROPOSED STUDY INITIATION/COMPLETION DATES

Initiation **October 5, 2018**

Completion **December 31, 2018**

DESCRIPTION OF STUDY OBJECTIVE

CW32A (EPA Registration No. 1677-[pending]) will be tested to determine food contact surface sanitizing efficacy against *Shigella flexneri* ATCC 29508, *Shigella sonnei* ATCC 11060, *Cronobacter sakazakii* ATCC 12868 and *Staphylococcus aureus* – CA-MRSA USA 400 ATCC BAA-1683 with the test parameters outlined below. AOAC 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants will be the test method utilized in making the sanitizing claim.

Test Parameters

Ecolab SOP number:	MS009; *Germicidal & Detergent Sanitizing Action of Disinfectants*
Test Systems:	*Shigella flexneri* ATCC 29508 *Shigella sonnei* ATCC 11060 *Cronobacter sakazakii* ATCC 12868 *Staphylococcus aureus* – CA-MRSA USA 400 ATCC BAA-1683
Exposure Time:	30 seconds
Exposure Temperature:	25±1°C
Test Substance Batches:	P081581 P081781
Test Substance Diluent:	500 ppm synthetic hard water
Test Substance Concentration:	0.25 oz/ gallon resulting in the active ingredient at or below the lower limit of 250 ppm Dodecylbenzene Sulfonic Acid (LAS).

Ecolab
Study Identification Number 1800076

TEST SUBSTANCE IDENTIFICATION

Test Substance Name: **CW32A**

Batch Identification: P081581
P081781

Formula Code: 919871

Date of Manufacture:

CW32A Batch Number	Date of Manufacture
P081581	August 15, 2018
P081781	August 17, 2018

An aliquot of the test substance batches will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, MN until the quality of the formula no longer affords evaluation. Test substance not dispersed for retention, chemical quality verification or efficacy testing will be stored in Ecolab Microbiological Services cabinet until disposed.

QUALITY ASSURANCE UNIT MONITORING

The protocol, pesticide efficacy in-life and final report are proposed to be inspected by the Ecolab Quality Assurance Unit (QAU) in accordance with their current standard operating procedures. The following proposed Ecolab QA inspections are for planning purposes only and may change. Ecolab QA inspections that are performed, along with their dates and auditors, will be included in the study final report. Changes in Ecolab QA inspections from those proposed below will not require revision of this protocol.

Proposed QAU Monitoring

Protocol Audit
Pesticide Efficacy In-Life Inspection
Final Report Audit

Ecolab
Study Identification Number 1800076

CHEMICAL QUALITY VERIFICATION

Proposed Experimental Start/Termination Dates

The chemical quality verification of the test substance concentrates was performed under Ecolab GLP study number 1800060. Experimental start and termination dates are documented within that study. The chemical quality verification on a single batch of test substance use-solution will be performed under Ecolab GLP study number 1800073. Experimental start and termination dates will be documented within that study.

Method

Chemical analysis was performed on each batch of the test substance concentrate to determine the concentration of the active ingredient under Ecolab GLP study number 1800060. Chemical analysis will be performed on a single batch of test substance use-solution prepared at 0.25 oz/gallon resulting in the active ingredient at or below the lower limit of 250 ppm Dodecylbenzene Sulfonic Acid (LAS) under Ecolab GLP study number 1800073. The dilution procedure for the test substance use-solution chemical analysis can be found in the Test Substance Concentration section of the protocol. The test substance use-solution for the chemical analysis will be prepared in sterile laboratory purified water.

The chemical quality verification will be performed by the Analytical Lab using the method listed below. The method has been deemed acceptable by the Analytical Lab and the study sponsor to ensure proper characterization of the test substance concentrate and the test substance use-solution. Statistical treatment of test results may be inherent to the method. Additional volumes and dilutions may be necessary to determine the chemistry of the use-solution sample.

QATM-216A; ***Determination of Lactic Acid, Citric Acid, and Sodium Citrate by HPLC***
Lactic acid, citric acid, and/or citrate content are determined using HPLC-UVD at 210 nm and external standard quantitation by peak area.

QATM-279; ***Anionic Content by Surfactant Electrode***
The surfactant electrode responds to the concentration of anionic surfactant in aqueous solution. Using a standardized cationic solution as titrant and the surfactant electrode to identify the endpoint, the concentration of anionic surfactant in an aqueous solution can be determined by titration.

The most current QATMs and product specific Bill of Quality will be used during the course of this study for the chemical and physical analysis.

Ecolab
Study Identification Number 1800076

Interpretation of Results

The concentration of the active ingredient in the test substance concentrates will be judged acceptable for pesticide efficacy testing when within the range specified by the Confidential Statement of Formula (CSF) upper and lower certified limits as seen in the table below.

Test Substance Concentrate Acceptance Limits		
Active Ingredient	**CSF Lower Certified Limit**	**CSF Upper Certified Limit**
Dodecylbenzene Sulfonic Acid (LAS)	11.4%	14.4%

The concentration of lactic acid in the test substance concentrates will be judged acceptable for pesticide efficacy testing when within the range specified by the Confidential Statement of Formula (CSF) upper and lower certified limits as seen in the table below.

Test Substance Concentrate Acceptance Limits		
Ingredient	**CSF Lower Certified Limit**	**CSF Upper Certified Limit**
Lactic Acid	29.5%	38.6%

The concentration of the active ingredient in the test substance use-solution at the lower limit is < 1%. Therefore the lower acceptance limit will be expanded by 10%. The expanded range is based on 40 CFR § 158.350 (Certified Limits) and was calculated as shown below.

Calculated Lower Acceptance Limit for Dodecylbenzene Sulfonic Acid
= [0.0250% - (0.0250 x 0.1)] = 0.0225%

The calculated upper acceptance limit for the active ingredient in the test substance use-solution was determined by adjusting 2% above the lower limit per U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Test Guidelines 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing (February 2018) as shown below.

Calculated Upper Acceptance Limit for Dodecylbenzene Sulfonic Acid
= [0.0250% + (0.0250 x 0.02)] = 0.0255%

The concentration of the active ingredient in the test substance use-solution will be judged acceptable for pesticide efficacy testing if within the expanded acceptance limit of 0.0225-0.0255% Dodecylbenzene Sulfonic Acid.

Ecolab
Study Identification Number 1800076

The concentration of Lactic Acid in the test substance use-solution at the lower limit is < 1%. Therefore the lower acceptance limit will be expanded by 10%. The expanded range is based on 40 CFR § 158.350 (Certified Limits) and was calculated as shown below.

Calculated Lower Acceptance Limit for Lactic Acid
= [0.0648% - (0.0648 x 0.1)] = 0.0583%

The calculated upper acceptance limit for Lactic Acid in the test substance use-solution was determined by adjusting 2% above the lower limit per U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Test Guidelines 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing (February 2018) as shown below.

Calculated Upper Acceptance Limit for Lactic Acid
= [0.0648% + (0.0648 x 0.02)] = 0.0661%

The concentration of Lactic Acid in the test substance use-solution will be judged acceptable for pesticide efficacy testing if within the expanded acceptance limit of 0.0583-0.0661%.

The Chemical Quality Verification results will be reported in the final report of this study.

PESTICIDE EFFICACY TESTING

Proposed Experimental Start/Termination Dates

Experimental Start Date	October 2018
Experimental Termination Date	October 2018

Methods

Pesticide efficacy data will be generated by the Microbiology Lab using the most current methods listed below. See the specific methods in the Protocol Appendix.

Method Number	Method Name
MS008	*Synthetic Hard Water Preparation & Standardization*
MS009	*Germicidal & Detergent Sanitizing Action of Disinfectants*
MS088	*Test Substance Use-Solution Preparation for Analysis*
MS111*	*Antibiotic Susceptibility Tests*

*For *Staphylococcus aureus* – CA-MRSA USA 400 ATCC BAA-1683

Ecolab
Study Identification Number 1800076

Test Method Requirement and Test System Justification

The following apply when determining the effectiveness of a non-halide food contact surface sanitizer; two samples, representing different batches are required to be tested for supplemental organisms. The organisms that will be tested are *Shigella flexneri* ATCC 29508, *Shigella sonnei* ATCC 11060, *Cronobacter sakazakii* ATCC 12868 and *Staphylococcus aureus* – CA-MRSA USA 400 ATCC BAA-1683. AOAC 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants for the above stated organisms is recommended based on the U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2300: Sanitizers for Use on Hard Surfaces– Efficacy Data Recommendations, September 04, 2012. Also, U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing (February 2018) applies to this study.

Test Method Justification

Ecolab Microbiological Services SOP MS009; *Germicidal & Detergent Sanitizing Action of Disinfectants* will be the test method utilized in this study.

Test Systems and Identification

The test systems which will be utilized for this procedure are *Shigella flexneri* ATCC 29508, *Shigella sonnei* ATCC 11060, *Cronobacter sakazakii* ATCC 12868 and *Staphylococcus aureus* – CA-MRSA USA 400 ATCC BAA-1683. Identification will be performed by observing the colony morphology and performing a Gram stain.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing will be performed on *Staphylococcus aureus* – CA-MRSA USA 400 ATCC BAA-1683 following Ecolab Microbiological Services SOP MS111; *Antibiotic Susceptibility Tests.*

Test System	Antibiotic Susceptibility Testing Details
Staphylococcus aureus – CA-MRSA USA 400 ATCC BAA-1683	Disk Diffusion Method with 1 µg Oxacillin

Statement of Proposed Statistical Method

None

Test Substance Diluent

500 ppm synthetic hard water prepared as described in Ecolab Microbiological Services SOP MS008; *Synthetic Hard Water Preparation & Standardization* will be the diluent.

Ecolab
Study Identification Number 1800076

Test Substance Concentration

Antimicrobial efficacy testing will be performed with **CW32A** diluted at 0.25 oz/gallon to at or below the lower limit of 250 ppm Dodecylbenzene Sulfonic Acid (LAS).

Active Ingredient	CSF Lower Certified Limit	Specific Gravity	Percent Dilution*	Resulting ppm of Active Ingredient
Dodecylbenzene Sulfonic Acid (LAS)	11.4%	1.126	0.195%	250 ppm

*Study proposed dilution: (0.25 oz/1 gallon) x (1 gallon/128 oz) x (100%) = 0.195%

Resulting ppm of active ingredient =

$$\left(\frac{\%ActiveatLCL}{100\%}\right)\left(\frac{\%Dilution}{100\%}\right)(\text{Specific Gravity})\,(10^6)$$

The following calculation will be used to ensure that the active ingredient is at or below the lower limit of 250 ppm Dodecylbenzene Sulfonic Acid in the test substance use-solution:

Dilution based on % active from QATM-279 analysis:

g of test substance batch in 600 g to yield 250 ppm Dodecylbenzene Sulfonic Acid =

$$\frac{(250\text{ ppm})(600\text{ g})(100\%)}{(10^6)\,(\%\text{ Active in batch})}$$

Antimicrobial efficacy testing will be performed with **CW32A** diluted at 0.25 oz/gallon to at or below the lower limit of 648 ppm Lactic Acid.

Ingredient	CSF Lower Certified Limit	Specific Gravity	Percent Dilution*	Resulting ppm of Active Ingredient
Lactic Acid	29.5%	1.126	0.195%	648 ppm

*Study proposed dilution: (0.25 oz/1 gallon) x (1 gallon/128 oz) x (100%) = 0.195%

Resulting ppm of active ingredient =

$$\left(\frac{\%ActiveatLCL}{100\%}\right)\left(\frac{\%Dilution}{100\%}\right)(\text{Specific Gravity})\,(10^6)$$

Ecolab
Study Identification Number 1800076

The following calculation will be used to ensure that Lactic Acid is at or below the lower limit of 648 ppm in the test substance use-solution:

Dilution based on % active from QATM-216A analysis:

g of test substance batch in 600 g to yield 648 ppm Lactic Acid =

$$\frac{(648\ \text{ppm})(600\ \text{g})(100\%)}{(10^6)(\%\ \text{Active in batch})}$$

The test substance use-solutions should be prepared as shown below in bold or with an equivalent dilution to ensure both the Dodecylbenzene Sulfonic Acid (LAS) and the Lactic Acid are at or below their lower limits for use in efficacy testing:

CW32A Batch Number	Concentration from Analysis	Test Substance Weight*: Diluent Weight*
P081581	% Anionic (QATM 279): 12.9%	1.16 g : 598.84 g
	% Lactic Acid (QATM 216A): 33.8%	**1.15 g : 598.85 g**
P081781	% Anionic (QATM 279): 12.9%	1.16 g : 598.84 g
	% Lactic Acid (QATM 216A): 33.8%	**1.15 g : 598.85 g**

Chemical analysis was performed under Ecolab GLP study number 1800060.
*Weights may vary by ± 0.03g.

Exposure Time/Temperature

The test systems will be exposed to the test substance for 30 seconds at 25 ± 1°C

Neutralizer Medium

9 mL DE Broth

Plating Medium

Tryptone Glucose Extract Agar

Incubation Time/Temperature

All plates are incubated for 24-48 hours at 35 ± 2°C with the exception of *Cronobacter sakazakii* ATCC 12868 which will be incubated for 24-48 hours at 30 ± 2°C

Ecolab
Study Identification Number 1800076

Test Controls

The following controls will be incorporated with the test procedure for each test system:

a. Initial Numbers Control
b. Neutralization Control
c. Test System Purity
d. Test Substance Diluent Sterility Control
e. Test Substance Sterility Control

Details on each of the above controls can be found in Ecolab SOP MS009 located in Protocol Appendix.

Interpretation of Test Results

The performance standard for a food contact sanitizer requires at least a 5 log reduction (≥99.999%) in the numbers of test system compared to the initial numbers control results within 30 seconds.

DATA RETENTION

Following the completion of the study, the original final report and raw data will be archived at the Ecolab Schuman Campus in Eagan, Minnesota or at an approved off-site location. All records that would be required to reconstruct the study and demonstrate adherence to the protocol will be maintained for the life of the commercial product plus four years.

TEST SUBSTANCE RETENTION

An aliquot of each batch of test substance will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, Minnesota until the quality of the formula no longer affords evaluation.

GOOD LABORATORY PRACTICES

This study will be conducted according to Good Laboratory Practices, as stated in 40 CFR Part 160. If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study. The sponsor will be notified as soon as it is practical whenever an event occurs that could have an effect on the validity of the study.

Ecolab
Study Identification Number 1800076

- **Name and Address of Sponsor**

  Kaitlin Lake
  Ecolab Schuman Campus
  655 Lone Oak Drive
  Eagan, MN 55121

- **Name and Address of Performing Laboratory**

  Ecolab Schuman Campus
  655 Lone Oak Drive
  Eagan, MN 55121

- **Name and Address of Study Director**

  Laurinda Holen
  Ecolab Schuman Campus
  655 Lone Oak Drive
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Kaitlin Lake 10.5.18
Sponsor Date

Laurinda Holen 10/5/18
Study Director Date

Ecolab
Study Identification Number 1800076

PROTOCOL APPENDIX

Microbiological Services (MS) Methods:

MS008	*Synthetic Hard Water Preparation & Standardization*
MS009	*Germicidal & Detergent Sanitizing Action of Disinfectants*
MS088	*Test Substance Use-Solution Preparation for Analysis*
MS111	*Antibiotic Susceptibility Tests*

ECOLAB
MICROBIOLOGICAL SERVICES

TITLE: **Synthetic Hard Water Preparation & Standardization**

NUMBER: **MS008-25**

EFFECTIVE: **04/01/16**

1.0 PURPOSE

To describe how to prepare standardized synthetic hard water solution to be used for diluting products that possess hard water claims.

2.0 SYNTHETIC HARD WATER PREPARATION

2.1 Fill out a media preparation sheet for Solution A and Solution B. Retain in the Media Preparation Log Book. Prepare 1 L of each solution or alternate amount with proportional ingredients.

2.2 Solution A Preparation

Magnesium Chloride ($MgCl_2 \cdot 6H_2O$)	67.74 ± .1 g
Calcium Chloride ($CaCl_2 \cdot 2H_2O$)	97.99 ± .1 g
Sterile Lab Purified Water	1 L

2.2.1 Dissolve powders in 600 mL of boiled lab purified water, and then bring to 1 L volume in a 1 L volumetric flask after solution has cooled.

2.2.2 Dispense into appropriate containers (for example, 250 mL Pyrex screw cap bottles) and autoclave for ≥ 15 minutes at ≥ 121°C.

2.2.3 Label using the standard Ecolab labels with a 1 month expiration date and store at 2 – 8°C.

2.2.4 Quality Control

2.2.4.1 Visual: Clear solution
2.2.4.2 Sterility Check: Sterile after incubation at 32 ± 2°C for ≥ 5 days
2.2.4.3 Expiration Date: One month at 2 – 8°C

2.3 Solution B Preparation

Sodium Bicarbonate ($NaHCO_3$)	56.03 ±.1 g
Sterile Lab Purified Water	1 L

TITLE: **Synthetic Hard Water Preparation & Standardization**

NUMBER: **MS008-25**

2.3.1 Dissolve in 600 mL of boiled lab purified water, then bring to 1 L volume in a 1 L volumetric flask with lab purified water after solution has cooled.

2.3.2 Filter sterilize through a 0.45 micron filter into appropriate sterile containers. (approximately 150 – 200 mL per container)

2.3.3 Label using the standard Ecolab labels with a one month expiration date and store at 2 – 8°C.

2.3.4 Quality Control

2.3.4.1 Visual: Clear solution
2.3.4.2 Sterility Check: Sterile after incubation at 32 ± 2° C for ≥ 5 days
2.3.4.3 Expiration Date: One month at 2 – 8° C

2.4 Hard Water Preparation

2.4.1 To avoid precipitation of the hard water solution, water should be at room temperature before the addition of Solutions A or Solution B.

Total hardness as ppm $CaCO_3$ = 2.495 × ppm Ca + 4.115 × ppm Mg

2.4.2 To each 1 L of water to be prepared add 1 mL of Solution A for each 100 ppm of $CaCO_3$ hardness desired plus 4 mL of Solution B (e.g. for 500 ppm synthetic hard water add 5 mL of Solution A and 4 mL of Solution B per liter of water).

2.4.3 Bring to 1 L volume with sterile lab purified water. If preparing more than 1 L, combine flasks in a sterile 4 L beaker blender after adding appropriate amounts of Solutions A and Solution B and bringing to volume.

2.5 Alternate Hard Water Preparation: Commercial Preparation

2.5.1 Use a commercially available standard, preferably NIST traceable, to prepare synthetic hard water (e.g. Hach Chemical Company 218710).

2.5.2 To prepare a 400 ppm as $CaCO_3$ solution, add four ampules of 10,000 ppm as $CaCO_3$ standard (10 mL each ampule) to a 1 L volumetric flask.

2.5.3 Add sterile lab purified water up to 1 L mark. Solutions of other water hardness and different volumes may be prepared as appropriate.

2.6 The pH of all test waters less than 2000 ppm hardness (as $CaCO_3$) should be 7.6 – 8.0. Adjustment of hard water pH using NaOH or HCl may be necessary depending on the starting water pH.

TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-25

3.0 STANDARDIZATION OF SYNTHETIC HARD WATER

3.1 Method Check – Prior to standardization of the synthetic hard water, the accuracy of the titration method must be checked by analyzing a 500 ppm $CaCO_3$ standard. This must be performed on a monthly basis or when testing new batches of Solution A and Solution B.

3.1.1 Dilute 10 mL of a 1000 ppm $CaCO_3$ standard (1 mL = 1 mg $CaCO_3$) in 10 mL of lab purified water to result in a 500 ppm $CaCO_3$ solution.

3.1.2 Dilute 10 mL of the 500 ppm $CaCO_3$ solution in 40 mL of lab purified water in a beaker.

3.1.3 Test solution as described in 3.2.2 – 3.2.5.

3.1.4 The hardness of the 500 ppm solution is determined as follows:

$$\text{hardness (ppm)} = (\text{mL EDTA}) \times 100$$

3.1.5 Record the result and the lot number of the standard on Form 3011. Hardness of the 500 ppm $CaCO_3$ solution must be 500 ± 20 ppm $CaCO_3$. Failure of the standard to fall within this range indicates a problem in the test method. Corrective actions should be documented in the comments section on Form 3011. The procedure may be used for standardization of synthetic hard water only when results of the standard are within the range described above.

3.1.6 Records from the current and previous year will be kept in the Microbiological Services Equipment Maintenance binder. All earlier records will be archived in the first quarter of the current year. For example, records from 2016 will be archived by March of 2018. Records will be transferred to Ecolab Archives at the Ecolab Schuman Campus in Eagan, MN or to an approved off-site location.

3.2 Sample Testing/Standardization

3.2.1 Dilute 10 mL of prepared hard water in 40 mL of lab purified water in a beaker.

3.2.2 Add 1 mL water hardness buffer with magnesium. Use hood when adding; the buffer has irritating vapors.

3.2.2.1 The buffer is VWR product code VW3491 (or equivalent)

TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-25

3.2.2.2 Approximate composition of buffer, % by weight:

Ammonia	56-57
Ammonium chloride	6-7
EDTA-Magnesium Tetraacetate Salt	0.5
Water	> 35

Note: This buffer has a relatively short expiration.

3.2.3 Optional: Add 1 mL inhibitor – needed only if previous titration without it has been unsatisfactory (refer to 3.2.5.2).

3.2.4 Add just enough Ecolab hardness indicator #016 to yield a pink coloration upon dissolving.

3.2.4.1 Hardness indicator 016 contains Calgamite (1-(1- hydroxy-4-methyl-2-phenylazo)-2-naphthol-4-sulfonic acid) as the actual indicator, along with inert ingredients.

3.2.4.2 It is obtained from Ecolab Test Kits (order through F&B Customer Service) at the Ecolab Engineering Center.

3.2.5 Add 0.01M EDTA slowly until the pink coloration turns blue. Record the number of milliliters of EDTA needed to create the color change.

3.2.5.1 The titration should be completed within five minutes of buffer addition to minimize tendency toward $CaCO_3$ precipitation.

3.2.5.2 If the end point color change is not clear and sharp (e.g. the color changes to blue and then drifts back to pink) then an inhibitor/complexing agent must be added (or possibly, the indicator has deteriorated).

3.2.5.3 Prepare inhibitor solution by dissolving 5.0 g sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$) or 3.7 g $Na_2S \cdot 5H_2O$ in 100 mL distilled water. Prepare and dispense in hood. This inhibitor solution deteriorates quickly though air oxidation and should be made each day it is needed.

3.2.5.4 Dilute new sample of test solution and re-titrate beginning with step 3.2.2, including addition of inhibitor.

3.2.6 The hardness of the water is determined as follows:

Hardness as mg $CaCO_3$ / L = (mL EDTA × 1000)/10 mL of sample
= mL EDTA × 100

3.2.7 Upon titration, hardness must not exceed 20 ppm above or below the ppm specified in test procedure/protocol/lab statement. Therefore, if a claim is for 500 ppm, the titration must yield 500 ± 20 ppm. If ppm hardness is out of the established range, the sample should be retitrated. Upon a second titration, if ppm hardness is still outside established ranges, the hard water must be diluted or additional solution added to yield the desired ppm. After adjustments have been made, the water must be titrated to determine ppm hardness.

3.2.8 Only two adjustments may be made to the hard water following the above procedure. If the hard water is outside the established limits after two adjustments, the water must be disposed of and the process reinitiated.

3.2.7 For GLP testing, record Hard Water Preparation and Standardization on Form 3010 or Form 3113.

4.0 RELATED FORMS

4.1 Form 3010: Synthetic Hard Water Preparation & Standardization
4.2 Form 3011: Water Hardness Standard Results
4.3 Form 3072: Solution A Prep Log
4.4 Form 3074: Solution B Prep Log
4.5 Form 3113: Test Substance Use-Solution Preparation for Analysis

5.0 REFERENCES

5.1 AOAC (2011) Method 960.09 (E)
5.2 APHA, Standard Methods for the Examination of Water & Wastewater, 21st Ed., 2005. Section 3500-Ca B. EDTA Titrimetric Method.

6.0 MOST RECENT REVISION SUMMARY

In 2.1, added option to prepare amount other than 1 L of Solution A or Solution B.

Prepared by: Lynda Grieves Date: 3/3/2016 3/10/16
Quality Assurance: [signature] Date: 03 Mar 2016
Management: Sherri St. Clair Date: 03 Mar 2016

ECOLAB
MICROBIOLOGICAL SERVICES

TITLE: **Germicidal & Detergent Sanitizing Action of Disinfectants**

NUMBER: **MS009-26**

EFFECTIVE: **10/19/17**

1.0 PURPOSE

To determine the efficacy of products used for sanitizing pre-cleaned, nonporous food contact surfaces. Additionally, this document describes the procedure to determine the efficacy of teat dips. The MS009 Attachment details the modifications required for teat dip efficacy testing.

2.0 CULTURE MEDIA – Propagation

2.1 AOAC Nutrient Broth
2.2 Nutrient Agar A (slants not made from pre-mixed dehydrated media)
2.3 Nutrient Agar B (plates)
2.4 Other media suitable for culturing specified test systems

3.0 SUBCULTURE MEDIA – Plating

3.1 Tryptone Glucose Extract Agar
3.2 D/E Agar
3.3 Brain Heart Infusion Agar
3.4 Tryptic Soy Agar (with or without 5% Sheep's Blood)
3.5 Other media suitable for culturing specified test systems

4.0 NEUTRALIZER

4.1 D/E Neutralizing Broth
4.2 Chambers Medium
4.3 0.1 – 0.5% Sodium thiosulfate
4.4 Letheen Broth
4.5 Other appropriate neutralizer

5.0 REAGENTS & APPARATUS

5.1 Phosphate Buffered Dilution Water (PBDW)
5.2 Phosphate Buffered Saline with 0.1% (v/v) Tween 80 (PBS & Tween 80)

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

5.3 Glassware

5.3.1 Sterile 250 mL Erlenmeyer flasks
5.3.2 Appropriately sized volumetric flasks
5.3.3 20 × 150 and 25 × 150 mm test tubes

5.4 Petri Dishes

5.4.1 Sterile disposable Petri dishes, 15 × 100 mm

5.5 Water Bath

5.5.1 Constant temperature water bath that can maintain a test temperature ± 1°C of required test temperature

5.6 Transfer Loops

5.6.1 Reusable metal or sterile plastic disposal transfer loops

5.7 Sterile Buchner Funnel (or equivalent) containing Whatman No. 2 Filter Paper

5.8 Sterile disposable 50 mL centrifuge tubes

5.9 Pipets/Transfer Device

5.9.1 Sterile disposable pipets
5.9.2 Micropipettor with sterile disposable tips

6.0 TEST SUBSTANCE

6.1 To test hard water tolerance, the test substance may be diluted in synthetic hard water (refer to MS008).

6.2 If dilution of the test substance is required, use greater than 1.0 mL or 1.0 g of product. The use-solution must be tested within three hours of preparation or within the known stability of the solution.

7.0 TEST SYSTEMS PREPARATION

7.1 Test Systems

7.1.1 *Staphylococcus aureus* ATCC 6538
7.1.2 *Escherichia coli* ATCC 11229
7.1.3 Refer to the MS009 Attachment for additional microorganisms that may be used to evaluate teat dip efficacy
7.1.4 Other test systems may be tested with appropriate culturing modifications

7.2 Defrost a single cryovial of frozen stock culture at room temperature and briefly vortex. Streak one loopful of the thawed frozen stock onto a Nutrient Agar A Slant and incubate at 35 ± 2°C for 24 ± 2 hours.

7.3 For the final test culture, add 5 mL PBDW to the inoculated slant. Use a sterile loop to dislodge growth from agar surface. Collect mixture and transfer to the balance of the 99 mL PBDW and mix. Inoculate a minimum of five Nutrient Agar B plates with 200 µL of the mixture to create a bacterial lawn. Incubate at 35 ± 2°C for 24 ± 2 hours.

7.4 Harvest the test system from the plates by adding a minimum of 5 mL of PBS + Tween 80 to each plate. It may be necessary to use less than 5 mL of PBS + Tween 80 to harvest some test systems in order to achieve the necessary titer. Use a sterile rod or equivalent to gently dislodge the culture from the agar surface. Combine the culture from all plates and mix thoroughly.

7.5 Filter the culture through sterile Whatman No. 2 filter paper contained in Buchner funnel (or equivalent). To accomplish this, place a sterile tube in the collection flask and assemble the Buchner funnel on top. Pre-wet the filter paper with about 1 mL of PBDW and initiate the vacuum to create a proper seal. Process the culture directly through the filter paper, under vacuum, collecting the test system suspension into the sterile tube. Once the culture is processed, remove the tube and vortex the suspension.

7.5.1 Adjust the density of the culture suspension, if necessary, by dilution using sterile PBDW to yield approximately $1.0 \times 10^9 - 1.0 \times 10^{10}$ organisms per milliliter.

7.5.2 1.0×10^9 organisms/mL corresponds roughly to % transmittance readings of 0.1% to 1.0%T at 580 nm.

7.5.3 Adjusting the density of the culture suspension may not be required for determining the efficacy of teat dips.

8.0 OPERATING TECHNIQUE

8.1 Refer to the MS009 Attachment for the operating technique suggested for determining the efficacy of teat dips.

8.2 Dispense 99 mL of test substance into a sterile 250 mL Erlenmeyer flask. Prepare a single flask for each test substance to be tested. Duplicate flasks should be evaluated during non-regulated or screening tests, where possible.

8.3 Also prepare a flask with 99 mL of sterile PBDW, per test system, for enumeration of initial numbers control and treat in the same manner as the test flasks.

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

8.4 Place flasks into a 25 ± 1°C temperature water bath (or other desired test temperature) and let equilibrate for ≥ 10 minutes.

8.5 Adding Test System to Test Substance

8.5.1 Swirl the test flasks, creating enough residual motion to prevent pooling of the test system.

8.5.2 While the liquid is still in motion, place the tip of the pipet containing the test system so it is partially immersed in the test substance midway between the center and edge of the flask. Add 1 mL of test system suspension to 99 mL of the test substance.

8.5.3 Avoid touching the sides of the flask with the pipet.

8.5.4 After addition of the inoculum, swirl the flask to thoroughly mix contents.

8.5.5 At the 30 second exposure period (or other exposure time(s) as appropriate), transfer a 1 mL portion of the test mixture to 9 mL of appropriate neutralizer and mix well. This corresponds to the 10^{-1} dilution. If necessary to achieve neutralization, a 99 mL aliquot of neutralizer may be used. This corresponds to the 10^{-2} dilution.

8.6 Plate 1 mL and 0.1 mL of the neutralized contents in quadruplicate. Single plating and additional dilutions may be performed if testing is not for regulatory purposes. Use the pour plate or spread plate technique and an appropriate subculture medium. For calculation purposes, when 9 mL of neutralizer is used, this corresponds to the 10^{-1} and 10^{-2} dilutions, respectively.

8.7 For the numbers control, add 1 mL of the test system suspension to 99 mL of PBDW in the same manner as done in the test. Within 30 seconds of addition of test system suspension, transfer 1 mL into 9 mL neutralizer (or 99 mL if used in the test) and mix well. This corresponds to the 10^{-1} or 10^{-2} dilution, respectively.

8.8 Make serial 10-fold dilutions in 9 mL of PBDW to 10^{-6}.

8.9 Plate 1 mL and 0.1 mL of the 10^{-6} dilution in quadruplicate using pour or spread plate technique using the subculture medium used in the test. For calculation purposes, this corresponds to the 10^{-6} and 10^{-7} dilutions, respectively.

Note: 1 mL aliquots may be split in half and plated over two plates.

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

9.0 CONTROLS

9.1 Neutralization Control

9.1.1 A neutralization confirmation test must be performed in advance or concurrently with the efficacy test.

9.1.2 Serially dilute the test system suspension to target between 10 – 100 CFU once the control is plated. When the target titer is 10^9 to 10^{10}, the 10^{-5} and 10^{-6} dilutions should provide an average of 10 – 100 CFU, once plated. Alternative dilutions may be used where appropriate.

9.1.3 Add 0.1 mL of the diluted test system suspension to inoculate each of the control tests in 9.1.4 and mix thoroughly. Test A should be inoculated within 30 seconds of preparation. After inoculation, each of the tests are held for a minimum of two minutes prior to plating. Plate 0.1 mL and 1.0 mL in duplicate. Use the pour plate or spread plate technique and the subculture medium used in the test.

9.1.4 Prepare the neutralizer control tests as shown in the table below:

Control Test	Description
A (Neutralizer Confirmation or NCT)	1 mL test substance to 9 or 99 mL neutralizer
B (Neutralizer Toxicity Treatment or NTT)	10 or 100 mL neutralizer
C (Test Culture Titer or TCT)	10 mL or 100 mL PBDW

9.2 Test System Purity

9.2.1 Inoculate the test system suspension onto Tryptic Soy agar with 5% sheep blood (e.g. Blood Agar plate) and streak for isolated colonies. If the test system does not grow on Blood Agar, use an alternate agar medium that supports growth.

9.2.2 Gram stain the test system.

9.3 Diluent Sterility

9.3.1 Plate 1.0 mL of the diluent using pour plate or spread plate technique.

TITLE: **Germicidal & Detergent Sanitizing Action of Disinfectants**

NUMBER: **MS009-26**

9.4 Test Substance Sterility

9.4.1 Plate 1.0 mL of the test substance using pour plate or spread plate technique.

Note: Neutralizer and PBDW sterility testing is conducted with routine media QC procedures.

10.0 INCUBATION

10.1 For regulated studies, incubate plates of *S. aureus* and *E. coli* at 35 ± 2°C for 24 – 30 hours.

10.2 For screening studies, incubate plates of *S. aureus* and *E. coli* at 35 ± 2°C for 2 – 3 days. Extended incubation helps to confirm the presence of sub-lethally injured cells that require extended incubation for recovery.

10.3 Incubate plates of other microorganisms at a time and temperature that provides adequate growth (e.g. 48 ± 4 hours).

11.0 DATA ANALYSIS

11.1 Enumerate and record plate counts as Colony Forming Units (CFU)/plate.

11.2 For initial numbers control and efficacy survivor counts determine the average CFU/mL as follows:

$$\text{Average CFU/mL} = \frac{(\text{Average CFU for } 10^{-x}) + (\text{Average CFU for } 10^{-y})}{10^{-x} + 10^{-y}}$$

Where 10^{-x} and 10^{-y} are the dilutions plated.

Use counts of 0 – 300 for calculation purposes. Score > 300 as TNTC (too numerous to count). If the average CFU/plate is < 1, then use < 1 when calculating the average CFU/mL.

11.3 Calculate mean $\log_{10}$ density for numbers controls plates. Calculate the mean $\log_{10}$ density for the treated sample plates. Calculate the $\log_{10}$ reduction for the treated sample:

Log_{10} reduction = mean $\log_{10}$ numbers control – mean $\log_{10}$ treated sample

Note: If duplicate flasks are tested, average the CFU/mL results for each flask to obtain a final average log reduction.

10/5/18

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

11.4 For the test to be considered valid, the numbers control must fall between 7.0 – 8.0 logs. For tests where the product meets the performance standard and the numbers control mean $\log_{10}$ density value is above 8.0, no retesting is necessary. For tests where the product fails to meet the performance standard and the numbers control mean $\log_{10}$ density is below 7.0, no testing is necessary.

11.5 In order to demonstrate effective neutralization of the sanitizer, the difference of A or B from C must be no more than 1.0 log.

11.6 Test system purity should have typical morphology for each of the organisms tested:

11.6.1 *Staphylococcus aureus* ATCC 6538: Medium to large, convex, circular, glistening, smooth, creamy, opaque, beta hemolytic – both light gold and darker gold colonies may be present on Blood Agar.

11.6.2 *Escherichia coli* ATCC 11229: Large, irregular, raised, gray and rough; greening of agar may be present on Blood Agar.

11.7 The diluent sterility control and test substance sterility control passes if there is no growth on the agar plate.

12.0 PERFORMANCE CRITERIA

12.1 In order for a sanitizer to be deemed effective, a 5 log reduction in the count of the number of microbes within 30 seconds is necessary. All test controls must also be valid.

13.0 RELATED FORMS

13.1 Form 3012: Germicidal & Detergent Sanitizing Action of Disinfectants
13.2 Form 3114: Germicidal & Detergent Sanitizing Action of Disinfectants – Teat Dip Bench Sheet

14.0 REFERENCES

14.1 MS008: Synthetic Hard Water Preparation & Standardization
14.2 MS040: Media Preparation & Storage – Media & Chemicals
14.3 Created from AOAC Method 960.09; Germicidal & Detergent Sanitizing Action of Disinfectants, 2013
14.4 EPA Good Laboratory Practice Standards, 40 CFR Part 160
14.5 US EPA OCSPP 810.2300: Sanitizers for Use on Hard Surfaces – Efficacy Data Recommendations, September 4, 2012

TITLE: **Germicidal & Detergent Sanitizing Action of Disinfectants**

NUMBER: MS009-26

15.0 MOST RECENT REVISION SUMMARY

General clarifications/text revisions added to 5.4.1, 7.1.4, 7.4, 8.5.4, 8.5.5, 8.6, 8.7, 8.9, 9.1 (title), 9.1.3, 9.4.1, 10.1 and 11.5. Added TSA/BAP to section 3.0. Revised 7.5 to clarify test organism procedure. Changed 8.2 to require the use of one flask for regulated testing and recommend two flasks for non-regulated/screening tests. Added note about splitting 1 mL aliquots after 8.9. Clarified text in 9.1.2 to recommend the 10-5 and 10-6 dilutions. Added note to 9.4. Added 10.2 (incubation of R&D studies for 2 –3 days). Added note in 11.3 to clarify how to calculate results from two flasks.

Prepared by: _____ Date: 10-18-17

Quality Assurance: _____ Date: 18 Oct 2017

Management: _____ Date: 18 Oct 2017 (10/18/18)

MS009 Attachment – 10/19/17

1.0 PURPOSE

To describe the modifications to MS009 required for determining the efficacy of teat dips.

2.0 TEST SYSTEMS

2.1 In addition to the microorganisms listed in step 7.1 in MS009, the following may be used for teat dip efficacy testing:

- *Streptococcus uberis* ATCC 27958
- *Streptococcus agalactiae* ATCC 27956
- *Streptococcus dysgalactiae* ATCC 27957
- *Pseudomonas aeruginosa* ATCC 15442
- *Klebsiella pneumoniae* ATCC 4352
- *Enterobacter aerogenes* ATCC 13048

3.0 OPERATING TECHNIQUE FOR TEAT DIP EFFICACY TESTING

3.1 Operating Technique without Milk (typically for pre-milking treatments)

3.1.1 Dispense 99 mL of test substance into a sterile 250 mL Erlenmeyer flask. Prepare duplicate flasks for each test substance and test system combination to be tested. Also prepare a flask with 99 mL of sterile PBDW for enumeration of initial numbers and treat in the same manner as the test flasks.

3.1.2 Swirl a test flask. While the test substance is still in motion, place the tip of the pipet containing the test system so it is partially immersed in the test substance midway between the center and edge of the flask. Add 1 mL of the test system suspension to 99 mL of the test substance. Avoid touching the sides of the flask with the pipet. Swirl the flask to thoroughly mix contents. After a 30 second exposure period, transfer 1 mL to 9 or 99 mL (or other volume as appropriate) of the appropriate neutralizer (based on inactivation of the test substance) and mix well immediately. Other exposure periods (e.g. 15 seconds) may be used. Because duplicate flasks are tested, duplicate plating of the test flasks is an acceptable alternative to quadruplicate plating.

Note: If the viscosity of the test substance does not allow for even distribution of the test system upon inoculation, the test flask may be swirled continuously throughout the exposure period. Alternately, a stir bar and stir plate may be used to aid in the distribution of the test system.

3.1.3 Enumerate microorganisms surviving treatment and initial numbers control as described in steps 8.7 – 8.9 in MS009.

MS009 Attachment – 10/19/17

3.1.4 Perform the neutralization controls as described in steps 9.1.1 – 9.1.4 in MS009.

3.1.5 Perform the test system purity control, diluent sterility control (if applicable) and the test substance sterility control as described in steps 9.2, 9.3 and 9.4 respectively in MS009.

3.1.6 Incubate plates as described in step 10.0 in MS009.

3.1.7 Perform data analysis as described in steps 11.1 – 11.3 in MS009.

3.1.8 For results to be considered efficacious, a 4 log reduction in the numbers of *Escherichia coli* and *Staphylococcus aureus* must occur within a 30 second exposure without milk.

3.1.9 For the test to be considered valid, the numbers control must fall between 7.0 – 8.0 logs. For tests where the product meets the performance standard and the numbers control value is above 8.0, no retesting is necessary. For tests where the product fails to meet the performance standards and the number control mean $\log_{10}$ density is below 7.0, no retesting is necessary.

3.1.10 Neutralization control, test system purity control and diluent sterility control analysis should be performed as described in steps 11.5, 11.6 and 11.7 in MS009.

3.2 Operating Technique with Milk (typically for post-milking treatments)

3.2.1 Dispense 90 mL of test substance into a sterile 250 mL Erlenmeyer flask. Prepare duplicate flasks for each test substance and test system combination to be tested. Also prepare a flask with 99 mL of sterile PBDW for enumeration of initial numbers and treat in the same manner as the test flasks.

3.2.2 Swirl a test flask. Add 10 mL of sterile milk, swirl well and remove 1 mL for a total of 99 mL in the test flask. This gives a 10% milk challenge. Immediately, swirl the test flask and, while the liquid is still in motion, place the tip of the pipet containing the test system so it is partially immersed in the test substance/milk mixture midway between the center and edge of the flask. Add 1 mL of culture to 99 mL of the test substance/milk mixture. Avoid touching the sides of the flask with the pipet. Swirl the flask to thoroughly mix contents. After a 30 second exposure period, transfer 1 mL to 9 or 99 mL (or other volume as appropriate) of the appropriate neutralizer (based on inactivation of the test substance) and mix well immediately.

Other exposure periods (e.g. 15 seconds) may be used.

MS009 Attachment – 10/19/17

Note: If the viscosity of the test substance does not allow for even distribution of the test system upon inoculation, the test flask may be swirled continuously throughout the exposure period. Alternately, a stir bar and stir plate may be used to aid in the distribution of the test system.

3.2.3 Enumerate microorganisms surviving treatment and initial numbers control as described in steps 8.7 – 8.9 in MS009.

3.2.4 Perform the neutralization controls as described in steps 9.1.1 – 9.1.4 in MS009.

3.2.5 Perform the test system purity control, diluent sterility control (if applicable) and the test substance sterility control as described in steps 9.2, 9.3 and 9.4 respectively in MS009.

3.2.6 Plate 1.0 mL milk using pour plate or spread plate technique. Milk sterility control passes if there is no growth on the milk sterility agar plate.

3.2.7 Incubate plates as described in step 10.0 in MS009.

3.2.8 Perform data analysis as described in steps 11.1 – 11.3 in MS009.

3.2.9 For results to be considered efficacious, a 4 log reduction in the numbers of the test system must be achieved in the exposure time with a milk challenge.

3.2.10 For the test to be considered valid, the numbers control must fall between 7.0 – 8.0 logs. For tests where the product meets the performance standard and the numbers control value is above 8.0, no retesting is necessary. For tests where the product fails to meet the performance standards and the number control mean $\log_{10}$ density is below 7.0, no retesting is necessary.

3.2.11 Neutralization control, test system purity control, diluent sterility control and test substance sterility control analysis should be performed as described in steps 11.5, 11.6 and 11.7 in MS009.

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MICROBIOLOGICAL SERVICES

TITLE: **Test Substance Use-Solution Preparation for Analysis**

NUMBER: **MS088-21**

EFFECTIVE: **06/15/18**

1.0 PURPOSE

To describe the preparation and active ingredient analysis of a diluted test substance (test substance use-solution). Use-solution analysis is included with pesticide efficacy studies, chemical quality verification studies and contract lab studies to verify that the active ingredient concentration in the use-solution corresponds to the dilution made for the claimed active ingredient(s).

2.0 PROCEDURE

2.1 Typically, use-solutions are prepared as follows –

2.1.1 Use-solutions are prepared at the Lower Certified Limit (LCL) or lower limit for efficacy studies.

2.1.1.1 The Lower Certified Limit refers to the lower level of active ingredient in the formulation (e.g. the concentrate or ready-to-use formula).

2.1.1.2 The Lower Limit refers to the lower level of active ingredient in the use-solution after a concentrate is adjusted.

2.2 Determine the concentration of active ingredient in the test substance concentrate to verify it is within claimed limits. Perform the analysis for each active ingredient in the product.

2.3 Prepare the test substance use-solution according to label instructions or as specified in protocol using diluent as described in 2.4. This use-solution should be labeled according to M032.

2.4 Deionized water may be used as the test substance diluent or the test substance diluent (e.g. hard/soft water or label instructed diluent) may be prepared in the same manner as used for pesticide efficacy testing.

Example: A 1:64 dilution is 1 part test substance, 63 parts diluent.

2.5 Analyze the test substance use-solution for active ingredient concentration using the same validated QATM that is, or will be, included in the finished good Bill of Quality (BOQ).

TITLE: Test Substance Use-Solution Preparation for Analysis

NUMBER: MS088-21

Note: The method used to measure active ingredient concentration in the use-solution may have limited sensitivity, accuracy and precision for quantitating the minimal levels of active ingredient found in many use-solutions. These factors may need to be considered when interpreting results. Any modifications to the QATM to adjust for this should be specified in the protocol.

2.6 Analyze the results. The active ingredient concentration in the use-solution should fall within the acceptance range as outlined in 3.5.

3.0 Formulas to Determine Use-solution Amounts and Acceptance Criteria

3.1 Dilution Factor (DF) Determination

3.1.1 Dilution Factor by Volume (DF_{vol})

Example: Dilution Factor (DF_{vol}) $= \left(\frac{1\text{ oz}}{1\text{ gallon}}\right)\left(\frac{1\text{ gallon}}{128\text{ oz}}\right) = 0.0078$

3.1.2 Density/Specific Gravity (SG) Calculation

When converting v/v dilutions to w/w, the specific gravity is applied. The specific gravity of water is treated as 1.0. Obtain density or specific gravity values for the test substance from confidential statement of formula (CSF) or suitable documentation. Convert as necessary to g/mL or unitless for SG.

Conversion Example:

$$\left(\frac{9.2\text{ lbs}}{\text{gallon}}\right)\left(\frac{1\text{ gallon}}{3785.412\text{ mL}}\right)\left(\frac{453.5924\text{ g}}{1\text{ lb}}\right) = 1.102\text{ g/mL}$$

$$\text{Density of Product} = \frac{\text{mass (g)}}{\text{volume (mL)}} \qquad \text{Specific Gravity} = \frac{\text{Density of Product}}{\text{Density of Water (1.0 g/mL)}}$$

$$\text{Density of Product} = 9.2\text{ lbs/gal} \sim 1.102\text{ g/mL} \qquad \text{Specific Gravity} = \frac{1.102\text{ g/mL}}{1.0\text{ g/mL}} = 1.102$$

3.1.3 $DF = (DF_{vol})(SG)$

$DF = (0.0078)(1.102) = 0.0086$

TITLE: **Test Substance Use-Solution Preparation for Analysis**

NUMBER: **MS088-21**

3.2 Use-solution prepared per label (e.g. 1000 g use-solution prepared at 1 oz/gallon dilution):

3.2.1 Target mass (g) of product = [Total use-solution mass (g)](DF)

Target mass (g) of product = (1000 g)(0.0086) = 8.6 g

3.2.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

Target mass (g) of diluent = 1000 g – 8.6 g = 991.4 g

3.2.3 Include a range of ± 0.03 g (~ 1 drop) or ± 0.3 g (~ 10 drops) to target masses when preparing use-solutions.

Note: any appropriate total use-solution mass may be used.

3.3 Use-solution prepared at CSF lower certified limit (LCL) – One active ingredient

3.3.1 Determine the active ingredient concentration (ppm) in the test substance use-solution when diluted (per label or protocol) using the test substance (concentrate) with active ingredient(s) at the LCL.

Example: 1 oz/gallon

$$\%\ \text{Dilution} = \left(\frac{1\ \text{oz Product}}{1\ \text{gallon}}\right)\left(\frac{1\ \text{gallons}}{128\ \text{oz}}\right)(100\%) = 0.781\%$$

$$\text{ppm active at LCL} = \left(\frac{\%\ \text{Active at LCL}}{100\%}\right)\left(\frac{\%\ \text{Dilution}}{100\%}\right)\left(\text{Specific Gravity} \times 10^{6}\right)$$

$$\text{Target mass (g) of product} = \frac{(\text{ppm Active at LCL})(\text{Total mass of use - solution})(100\%)}{(10^{6})(\%\ \text{Active Ingredient Result})}$$

3.3.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

Note: any appropriate total use-solution mass may be used.

3.4 Use-solution prepared from CSF lower certified limit (LCL) – multiple active ingredients

- Ensure that all active ingredients are at or below the calculated lower limit.
- This can be determined by calculating all active ingredient amounts and using an amount (of product) that ensures all active ingredients present to be less than or equal to the calculated lower limit.

TITLE: Test Substance Use-Solution Preparation for Analysis

NUMBER: MS088-21

3.4.1 Follow 3.3 to determine target masses (g) of product and diluent.

Note: any appropriate total use-solution mass may be used.

3.5 Determining the lower acceptance limit: The lower acceptance limit, which is the acceptable lower end of the lower limit, follows the guidance in 40 CFR Part 158.350:

If the target Lower Limit concentration (L) is (% by wt.)	Lower Acceptance Limit
$L \leq 1.0\%$	L – 10%
$1.0\% < L \leq 20.0\%$	L – 5%
$20.0\% < L \leq 100.0\%$	L – 3%

3.5.1 Example: Product diluted at 1 oz/gallon (product/diluent) for LCL dilution use-solutions

Where: CSF LCL = 16.43%; DF = 0.0086; Nominal (N) = 17.29%

Lower Limit= (CSF LCL)(DF) = (16.43%)(0.0086) = 0.141%

Lower Acceptance Limit can be up to 5% lower or
0.141% - 0.007% = 0.134% providing a target of 0.134-0.141%.

3.5.2 For products with multiple active ingredients, the concentrate is diluted so that all active ingredients are at or below the lower limit. As a result of diluting to ensure all active ingredients present are equal to or less than the lower limit, it is possible that some active ingredients may fall below the lower end of the range. It may be acceptable if this occurs. For products with more than one A.I., when analytical methods cannot differentiate between different A.I.s in a formulation, the individual LCL values may be added together to determine the lower certified limit of the total.

3.6 Determining the upper acceptance limit:
Because of difficulties associated with generating test samples exactly at the lower limit, the EPA has provided an acceptable range above the limit, the upper acceptance limit, described in 810.2000 as follows:

If the Lower Limit concentration (L) for the ingredient is (% by wt.)	The tested value may be above the lower limit by:
$L \leq 1.0\%$	L+2.0%
$1.0\% < L \leq 20.0\%$	L+1.0%
$20.0\% < L \leq 100.0\%$	L+0.6%

3.6.1 Using this approach, a product that has an active ingredient with a nominal concentration of 7.00% and LCL of 6.65% (based on 40 CFR Part 158.350), would have a testing allowance of up to 6.72%. In this example, the nominal concentration is greater than 1.0% and less than 20%, therefore, the appropriate testing range would be up to 1.0% above the LCL of 6.65% (6.65 +0.0665 = 6.7165, rounded to 3 significant figures = 6.72%).

4.0 RELATED FORMS

4.1 Form 3113: Test Substance Use-Solution Preparation for Analysis

5.0 REFERENCES

5.1 M032: Labeling Requirements
5.2 40 CFR 158.350
5.3 U.S. EPA OCSPP 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing (February 2018).

6.0 MOST RECENT REVISION SUMMARY

Added 2.1.1.1 and 2.1.1.2 and removed 2.1.2 as redundant. Clarified the purpose of using the specific gravity in 3.1.2. Revised section 3.5 and 3.6 to match current guidance used to determine the LCL. Added reference 5.3.

Prepared by: _____ Date: 6-11-18
Quality Assurance: _____ Date: 6/12/18
Management: _____ Date: 6-12-18

6/12/18
EXACT COPY

ECOLAB
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TITLE: Antibiotic Susceptibility Tests

NUMBER: MS111-08

EFFECTIVE: 07/01/18

1.0 PURPOSE

To verify the antibiotic susceptibility data of test systems. Test systems exhibiting resistant or intermediate antibiotic susceptibility should be confirmed prior to or in conjunction with culture maintenance storage and during the course of a study in which an antibiotic resistant or intermediate test system is tested.

2.0 CULTURE MEDIA

2.1 AOAC Nutrient Broth
2.2 AOAC Synthetic Nutrient Broth
2.3 Brain Heart Infusion Broth
2.4 Other medium suitable for culturing specified test systems

3.0 SUBCULTURE MEDIA

3.1 Mueller Hinton Broth
3.2 Mueller Hinton Agar
3.3 Tryptic Soy Agar with 5% Sheep's Blood
3.4 Tryptic Soy Agar
3.5 Other medium suitable for culturing specified test systems

4.0 REAGENTS & APPRATUS

4.1 0.85% Sterile Saline
4.2 Antibiotics (Oxacillin, Cefoxitin, Vancomycin, etc.)
4.3 Sterile Test Tubes 13 × 100 mm (or equivalent)
4.4 Antibiotic Susceptibility Disks
4.5 Antibiotic Susceptibility Disk Dispensers (optional)
4.6 Sterile Disposable Petri Dishes, 15 × 100 mm
4.7 Transfer Loops: Suitable metal or plastic disposable transfer loops
4.8 Vortex Mixer
4.9 Pipets
4.10 50 mM $ZnSO_4$

TITLE: Antibiotic Susceptibility Tests

NUMBER: MS111-08

5.0 TEST SYSTEM PREPARATION

5.1 Transfer the organism as described in the efficacy test method. Alternately, quality control (QC) organisms may be transferred a minimum of three consecutive transfers, but less than 15 total, in the appropriate broth medium before using to inoculate for testing.

5.2 If only one transfer is missed per seven day period, it is not necessary to repeat the three consecutive transfers, unless otherwise stated in applicable efficacy method.

5.3 If two or more transfers are missed, repeat with three consecutive transfers, unless otherwise stated in applicable efficacy method.

5.4 Transfers can be made on a daily, two day or other appropriate schedule depending on the growth requirements of the test system. Incubate at a temperature that provides good growth.

5.5 From the third or greater consecutive transfer, inoculate Tryptic Soy Agar with 5% Sheep's Blood (BAP) or other non-selective agar medium with a 24 ± 4 hour culture. If using the transfer schedule from an efficacy test, then BAP (or other non-selective agar) should be inoculated with a culture that could be used to inoculate a culture for an efficacy test.

6.0 INOCULUM PREPARATION

6.1 Turbidity Standard for Inoculum Preparation

6.1.1 To prepare a 0.5 McFarland Standard, add 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2 \cdot 2H_2O$) to 99.5 mL of 0.18 mol/L (0.36N) H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. Verify the density by measuring absorbance at 625 nm. The absorbance should be between 0.08 and 0.13 for a 0.5 McFarland Standard. Store the solution in tightly sealed tubes (same as those used to standardize the bacterial inoculum) in the dark at room temperature. Re-check the density monthly.

6.1.2 Alternately, a 0.5 McFarland Standard may be purchased from a lab supply company.

6.1.3 Measuring the absorbance of the bacterial inoculum at 625 nm in a spectrophotometer is another acceptable method of obtaining an inoculum of 0.5 McFarland Standard. The absorbance should be between 0.08 and 0.13.

TITLE: Antibiotic Susceptibility Tests

NUMBER: MS111-08

6.2 Colony Suspension Method

6.2.1 Inoculate sterile 0.85% saline or sterile broth with isolated colonies from an 18 – 24 hour incubated non-selective agar plate (refer to 5.5).

6.2.2 Adjust the suspension to achieve a turbidity equivalent to a 0.5 McFarland Turbidity Standard or equivalent. This should result in a suspension containing approximately 1×10^8 to 2×10^8 CFU/mL.

6.2.3 To measure the turbidity of the bacterial inoculum, use either a spectrophotometer (refer to 6.1.3) or perform visually by comparing the inoculum tube and the 0.5 McFarland Standard against a white background with contrasting black lines.

6.2.4 This method is recommended for testing most organisms including fastidious organisms and staphylococci for methicillin or oxacillin resistance.

6.3 Broth Culture Method

6.3.1 Select at least three to five isolated colonies of the same morphology from an agar plate (refer to 5.5). Touch the top of each colony with a sterile loop or swab and transfer into a tube containing 4 – 5 mL of a suitable broth medium.

6.3.2 Incubate the broth cultures at 35 ± 2°C for 2 – 6 hours or until it achieves or exceeds the 0.5 McFarland Standard.

6.3.3 Adjust the turbidity of the broth culture with sterile 0.85% saline or broth to achieve a turbidity equivalent to that of a 0.5 McFarland Standard.

6.3.4 To measure the turbidity of the bacterial inoculum, use either a spectrophotometer (refer to 6.1.3) or perform visually by comparing the inoculum tube and the 0.5 McFarland Standard against a white background with contrasting black lines.

6.3.5 This method may be used when colony growth is difficult to suspend directly and cannot be made smooth. It may also be used for non-fastidious organisms, with the exception of staphylococci, when 24 hour colonies are not available.

11/6/18

TITLE: Antibiotic Susceptibility Tests

NUMBER: MS111-08

7.0 DISK DIFFUSION METHOD

7.1 Pour petri dishes with Mueller Hinton agar or other appropriate medium. Plates should be poured to 4 mm thickness on a level, horizontal surface. Alternatively, commercially prepared Mueller Hinton Agar may be used. If moisture is present on the surface of the agar, dry the plate in a 35 ± 2°C incubator or in a laminar flow hood at room temperature with the lids cracked until dry (typically 10 – 30 minutes). Remove the disk cartridges from the refrigerator (2 - 8°C) or freezer (≤ -10°C) 1 – 2 hours before use to equilibrate.

Note: Mueller Hinton Agar is not an appropriate agar for all organisms. Alternate media may be used for those organisms.

7.2 Within 15 minutes of adjusting the turbidity of the inoculum (steps 6.2.3 or 6.3.3), dip sterile cotton swab into the adjusted suspension and remove excess liquid from the swab by rotating the swab and pressing firmly on the inside wall of tube. Never streak plates with undiluted overnight broth cultures or other unstandardized inocula.

7.3 Inoculate the dried agar plate by streaking the swab over the entire agar surface of the plate. Rotate the plate approximately 60° and steak the swab over the entire agar surface. Rotate another 60° and streak with the swab again. Finally, swab the rim of the agar.

7.4 Allow any excess surface moisture on the agar plate to dry. This may be done by leaving the lid of the plate ajar for three to five minutes in a biological safety cabinet. The agar plates should dry for no more than 15 minutes.

7.5 Dispense the antibiotic susceptibility disks onto the inoculated surface of the agar. The disks should be gently tapped to ensure contact is made with the surface of the agar. Disks should be placed at least 24 mm from each other (center of one disk to the center of another disk). Test three disks for each test and QC organism. Ensure that disks are not too close to the side of the Petri plate. Never move a disk once it has been placed on the surface.

7.6 Multiple drug resistant organisms (MDRO) are defined as organisms (predominantly bacteria) that are resistant to one or more classes of antibiotics.

7.7 Repeat 7.1 – 7.6 with the appropriate control organism(s) for the tested antibiotic listed in the table below. The control organisms are transferred for use in the test as described in 5.0. Acceptable zones of inhibition for each control organism are listed in the table below. The antibiotics listed in the table are frequently tested but it is not an exhaustive list. CLSI method M02 and M100 have a more extensive list of antibiotics and control organisms for antibiotic susceptibility testing of fastidious organisms.

11/6/18

TITLE: Antibiotic Susceptibility Tests

NUMBER: MS111-08

Acceptable Limits (in mm) for Quality Control strains used to monitor accuracy; nonfastidious organisms using Mueller-Hinton Agar.

Antimicrobial Agent	Disk Content	*Escherichia coli* ATCC 25922	*Staphylococcus aureus* ATCC 25923	*Pseudomonas aeruginosa* ATCC 27853
Cefoxitin	30 µg	23 – 29	23 – 29	–
Ceftazidime	30 µg	25 – 32	16 – 20	22 – 29
Gentamicin	10 µg	19 – 26	19 – 27	17 – 23
Imipenem	10 µg	26 – 32	–	20 – 28
Meropenem	10 µg	28 – 35	29 – 37	27 – 33
Oxacillin	1 µg	–	18 – 24	–
Vancomycin	30 µg	–	17 – 21	–

7.8 Within 15 minutes of disk application, invert the plates and incubate for 16 – 18 hours at 35 ± 2°C. Methicillin, oxacillin and nafcillin resistant staphylococci should be incubated for at least 24 hours before reading results. Cefoxitin resistant coagulase negative staphylococci are incubated for 18 – 24 hours. Coagulase negative staphylococci must be incubated for at least 24 hours if not resistant at 18 hours. Vancomycin resistant enterococci are incubated for 24 – 28 hours before reading results.

7.9 Interpret the sizes of the zones of inhibition by measuring the zone diameter (include the disk in the measurement) and comparing the value to the table in the MS111 Attachment or the package insert from the disk manufacturer if different from the MS111 Attachment, to determine susceptible, intermediate or resistance. The zone margin is the area showing no obvious visible growth that can be detected with the unaided eye. Some media or organisms may require illumination with reflected light source to read the zones. If no zone is present the result is the diameter of the disk.

Note: The disk diffusion method should not be used to determine the intermediate susceptibility or resistance of *Staphylococcus aureus* to vancomycin. Methicillin resistance can be tested with oxacillin MIC or cefoxitin disks. Cefoxitin is preferred for *S. aureus*.

TITLE: Antibiotic Susceptibility Tests

NUMBER: MS111-08

8.0 MINIMUM INHIBITORY CONCENTRATION (MIC) METHOD

8.1 Antibiotic Standard Solution Preparation

8.1.1 Antibiotics in powdered form should be obtained. Acceptable sources for the antibiotics include drug manufacturers, United States Pharmacopoeia or other commercial sources. Acceptable powders will have a label or certificate of analysis that states the generic drug name, lot number, potency and expiration date.

8.1.2 Use either of the formulae below to determine the amount of powder or diluent needed to prepare a standard solution. The standard solution concentration will typically be at least 1000 µg/mL or ten times the highest concentration to be tested, whichever is greater.

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Concentration (µg/mL)}}{\text{Potency (µg/mg)}}$$

or

$$\text{Volume (mL)} = \frac{\text{Weight (mg)} \times \text{Potency (µg/mg)}}{\text{Concentration (µg/mL)}}$$

Note: Potency, if not included on the certificate of analysis, can be determined using the following equation:
Potency = (assay purity) (active fraction) (1 – water content)

8.1.3 At least 10 mg of powder should be used to prepare the standard.

8.1.4 The standard solution may be filter sterilized, however it is not required since microbial contamination of antibiotic standard solutions is rare. Filter sterilized solutions can be dispensed in small volumes into sterile cryovials or sterile centrifuge tubes and stored for up to 6 months at ≤ -70°C. Vials may be thawed as needed and used on the date thawed. Unused stock must be discarded at the end of the day.

8.2 Macrodilution Procedure

8.2.1 Use 13 × 100 mm sterile test tubes.

8.2.2 Prepare test tubes as outlined in the table in 8.2.6. Alternate volumes may be used to create the desired dilutions/concentrations, but test tubes must have at least 1 mL as a final volume.

6/16/18

8.2.3 Within 15 minutes of adjusting the turbidity of the inoculum to 0.5 McFarland Standard (steps 6.2.3 or 6.3.3), dilute the adjusted inoculum 1:150 in sterile broth (Mueller Hinton Broth or other appropriate medium).

8.2.4 Within 15 minutes of step 8.2.3, add 1 mL of the diluted inoculum to each test tube prepared in 8.2.2. There should be approximately 5×10^5 CFU/mL test system in the test tubes.

8.2.5 Controls should be performed as follows:

- Positive control: Broth medium with 1 mL of diluted inoculum
- Negative control: Un-inoculated broth medium
- Colony count of inoculum suspensions: Results should be approximately 5×10^5 CFU/mL
- Test system purity: Streak diluted inoculum to appropriate agar medium

8.2.6 Incubate for 16 – 20 hours at 35 ± 2°C. Evaluations for Vancomycin resistance of *Enterococcus* species should be incubated for 24 – 28 hours.

Antibiotic Solution

Step	Concentration (µg/mL)	Source	Volume (mL)	Volume of Broth (mL)	Final Concentration (µg/mL)	Volume of Inoculum (mL)
1	5120	Stock	1	9	512	1
2	512	Step 1	1	1	256	1
3	512	Step 1	1	3	128	1
4	512	Step 1	1	7	64	1
5	64	Step 4	1	1	32	1
6	64	Step 4	1	3	16	1
7	64	Step 4	1	7	8	1
8	8	Step 7	1	1	4	1
9	8	Step 7	1	3	2	1
10	8	Step 7	1	7	1	1
11	1	Step 10	1	1	0.5	1
12	1	Step 10	1	3	0.25	1
13	1	Step 10	1	7	0.125	1

8.3 Results Interpretation

8.3.1 The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the test system in the tubes or microtiter wells detected by the unaided eye. For a test to be considered valid there must be growth in the positive control tube.

8.3.2 The presence of the test system must be confirmed in the test tube of the highest concentration with growth. The growth should be subcultured to appropriate media, incubated for 24 ± 4 hours at 35 ± 2°C, and Gram stained.

8.3.3 *Staphylococcus aureus* and Oxacillin – Results that are ≥ 4 µg/mL should be classified as resistant, and ≤ 2 µg/mL are susceptible.

8.3.4 *Staphylococcus aureus* and Vancomycin – Results that are ≥ 16 µg/mL should be classified as resistant, 4 – 8 µg/mL have reduced susceptibility (intermediate) and ≤ 2 µg/mL are susceptible.

9.0 MODIFIED HODGE TEST TO VERIFY CARBAPENEMASE PRODUCTION

9.1 Prepare a 0.5 McFarland standard suspension of *Escherichia coli* ATCC 25922, transferred as described in section 5.0 with an 18 – 24 hour culture used to make suspension, in broth or 0.85% saline. Then dilute 1:10 in broth or 0.85% saline.

9.2 Inoculate a Mueller Hinton Agar plate with a diluted suspension prepared as described in 7.2 and 7.3.

9.3 Allow the inoculated plate to dry for 3 – 10 minutes.

9.4 Place one ertapenem (10 µg) or meropenem (10 µg) disk on the inoculated agar plate.

9.5 Using a loop or swab, pick 3 – 5 colonies of the test or Quality Control (QC) organism from an 18 – 24 hour culture on Tryptic Soy Agar with 5% Sheep Blood or other non-selective medium. Streak growth in a straight line from the edge of the disk out. The streak should be at least 20 mm in length.

9.6 Incubate at 35 ± 2°C for 16 – 20 hours.

9.7 Results interpretation

9.7.1 Examine agar plate for enhanced growth around the test or QC organism streak at the intersection of the streak and zone of inhibition. The enhanced growth will have an appearance of a clover leaf shape.

9.7.2 Enhanced growth around a test or QC organism streak is a positive test for Carbapenemase production. No enhanced growth is negative for Carbapenemase production.

9.7.3 QC organisms are *Klebsiella pneumoniae* ATCC BAA-1705: Modified Hodge Test positive and *Klebsiella pneumoniae* ATCC BAA-1706: Modified Hodge Test negative.

10.0 EXTENDED-SPECTRUM β-LACTAMASE TEST FOR *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca & Proteus mirabilis*

10.1 Prepare a 0.5 McFarland standard suspension of the test system as described in section 5.0. An 18 – 24 hour culture should be used to make the inoculum suspension in broth or 0.85% saline.

10.2 Prepare a 1000 µg/mL stock solution of clavulante (either freshly prepared or from small aliquots stored frozen at ≤ -70°C). Add 10 µL of the clavulanate stock solution to disks of ceftazidime (30 µg) and cefotaxime (30 µg) disks. Disks should be allowed to absorb for approximately 30 minutes and should be dry enough for application. Disks must be used within one hour of preparation (addition of clavulanate to disks).

10.3 Prepare Mueller Hinton Agar plates or other appropriate medium as described in 7.1.

10.4 Inoculate agar plates as described in 7.3 – 7.4. Place disks prepared in 10.2 on inoculated plates as described in 7.5. Also test the antibiotic disks without the added clavulanate for comparison purposes. Incubate inverted at 35 ± 2°C for 16 – 18 hours. Extended-spectrum β-lactamase activity is determined when there is a ≥ 5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanate vs the zone diameter of the agent when tested alone.

10.5 Repeat 10.4 with the following quality control organisms – *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 and incubate with test system plates. *E. coli* ATCC 25922 must have ≤ 2 mm increase in zone diameter for antimicrobial agent testsed in combination with clavulanate vs the zone diameter when tested alone. *K. pneumoniae* ATCC 700603 must have ≥ 5 mm increase in zone diameter of ceftazidime-claulanate vs ceftazidime alone, and ≥ 3 mm increase in zone diameter of cefotaximine-clavulante vs cefotaxime alone.

11.0 METALLO-β-LACTAMASE PRODUCTIONS (RE-MODIFIED HODGE TEST)

11.1 Prepare a 0.5 McFarland standard suspension of *Escherichia coli* ATCC 25922, transferred as described in section 5.0 with an 18 – 24 hour culture used to make the suspension, in broth or 0.85% saline.

11.2 Inoculate a Mueller Hinton Agar plate with the *E. coli* ATCC 25922 suspension as described in 7.2 and 7.3. Allow plate to dry for 3 – 10 minutes.

TITLE: Antibiotic Susceptibility Tests

NUMBER: MS111-08

11.3 Using a loop or swab, pick 3 – 5 colonies of the test system or QC organism (*K. pneumoniae* ATCC BAA-1705 – positive control and *K. pneumoniae* ATCC BAA-1706 – negative control) from an 18 – 24 hour culture on Tryptic Soy Agar with 5% Sheep Blood or other non-selective medium. Streak the growth in a straight line from the center of the plate out to the periphery of the plate. The streak should be at least 20 mm in length. Allow to stand for 15 minutes.

11.4 Add 10 µL of 50 mM of ZnSO4 to an imipenem (10 µg) desk. Place the disk at the center of the inoculated agar plate. Incubate at 35 ± 2°C for 24 ± 4 hours.

11.5 Results interpretation

11.5.1 Examine the agar plate for enhanced growth around the test system or QC organism streak at the intersection of the streak and zone of inhibition. The enhanced growth will have an appearance of a clover leaf shape.

11.5.2 Enhanced growth around a test system or QC organism streak is a positive test for metallo-β-lactamase production. No enhanced growth is negative for metallo-β-lactamase production.

11.5.3 QC organism results – *K. pneumoniae* ATCC BAA-1705 should demonstrate enhanced (positive) growth and *K. pneumoniae* ATCC BAA-1706 should demonstrate non-enhanced (negative) growth.

11.5.4 Metallo-β-lactamase production is used to indicate the gene expression of NDM-1.

12.0 RELATED FORMS

12.1 Form 3142: Antibiotic Susceptibility Testing – Disk Diffusion Method
12.2 Form 3143: Antibiotic Susceptibility Testing – Minimum Inhibitory Concentration
12.3 Form 3156: Antibiotic Susceptibility Testing – Modified Hodge Test
12.4 Form 3163: Antibiotic Susceptibility Testing – Re-Modified Hodge Test Method

TITLE: **Antibiotic Susceptibility Tests**

NUMBER: **MS111-08**

13.0 REFERENCES

13.1 Clinical & Laboratory Standards Institute Method M02: Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard 13th Edition. January 2018

13.2 Clinical & Laboratory Standards Institute Method M07: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard 11th Edition. January 2018

13.3 Clinical & Laboratory Standards Institute Method M100-S28: Performance Standards for Antimicrobial Susceptibility Testing; 28th Informational Supplement. January 2018

13.4 Lee, K. et al. Evaluation of the Hodge Test and the Imipenem-EDTA Double Disk Synergy Test for Differentiating Metallo-β-lactamase Producing Isolates of *Pseudomonas* species and *Acinetobacter* species. Journal of Clinical Microbiology, October 2003 page 4623 – 4629

13.5 Siegel, J, et al, Management of Mult-Drug Resistant Organisms in Healthcare settings, 2006, CDC. Accessed https://www.ced.gov/ingectioncontrol/pdf/guideline/mdro-guidelines.pdf on 18June2018

14.0 MOST RECENT REVISION SUMMARY

- Updated for Clarification: 5.1, 6.2, 6.3, 7.7, 7.8, 8.1.1
- Added Information: 7.1, 7.5, 7.9, 8.1.2, 8.1.4
- Updated References: 13.1, 13.2, 13.3
- Added new sections: 7.6, 13.5

Prepared by: _____ Date: 6/22/18

Quality Assurance: _____ Date: 26 June 2018

Management: _____ Date: 6-26-18

MS111 Attachment – 07/01/18

From CLSI M100-S28: Performance Standards for Antimicrobial Susceptibility testing; 28th Informational Supplement, January 2018.

Use the table to determine resistance, intermediate and susceptibility levels for various disk antibiotics. If this table differs from the package insert from the manufacturer, then use the levels provided by the manufacturer.

Test System (Genus/Species or Family)	Antibiotic Disks & Zones of Inhibition (mm)						
	Ceftazidime 30 µg	Gentamicin 10 µg	Imipenem 10 µg	Meropenem 10 µg	Cefoxitin 30 µg	Vancomycin 30 µg	Cefuroxime 30 µg
Staphylococcus sp.	No criteria	R = ≤ 12 I = 13 – 14 S = ≥ 15	No criteria	No criteria	R = ≤ 21 S = ≥ 22	No criteria	No criteria
Enterococcus sp.	No criteria	No criteria	No criteria	No criteria	No criteria	R = ≤ 14 I = 15 – 16 S = ≥ 17	No criteria
Acinetobacter sp.	R = ≤ 14 I = 15 – 17 S = ≥ 18	R = ≤ 12 I = 13 – 14 S = ≥ 15	R = ≤ 18 I = 19 – 21 S = ≥ 22	R = ≤ 14 I = 15 – 17 S = ≥ 18	No criteria	No criteria	No criteria
Enterobacteriaceae	R = ≤ 17 I = 18 – 20 S = ≥ 21	R = ≤ 12 I = 13 – 14 S = ≥ 15	R = ≤ 19 I = 20 – 22 S = ≥ 23	R = ≤ 19 I = 20 – 22 S = ≥ 23	R = ≤ 14 I = 15 – 17 S = ≥ 18	No criteria	R = ≤ 14 I = 15 – 17 S = ≥ 18
Pseudomonas aeruginosa	R = ≤ 14 I = 15 – 17 S = ≥ 18	R = ≤ 12 I = 13 – 14 S = ≥ 15	R = ≤ 15 I = 16 – 18 S = ≥ 19	R = ≤ 15 I = 16 – 18 S = ≥ 19	No criteria	No criteria	No criteria

sp. = Species
No criteria = No criteria was listed in M100-S28 for these organism/antibiotic combinations. Consult manufacturer instructions
R = Resistant
I = Intermediate
S = Susceptible

11/6/18

Regulated Study Protocol Amendment

Study Title:	CW32A Supplemental Food Contact Sanitizing Efficacy
Study Number:	1800076
Amendment Number:	1800076-1A
Amendment Effective Date:	November 6, 2018

Description of Amendment

The protocol is being amended to clarify that the test substance use-solution chemical analysis was performed on CW32A batch P081781.

The protocol is being amended to clarify why chemical analysis was performed on lactic acid when the common practice is to only perform chemical analysis on the active ingredients. The analysis of lactic acid was performed due to a pending decision from the EPA on whether lactic acid is an active ingredient or not. Chemical analysis will be performed on both LAS and lactic acid in the event that lactic acid is determined to be an active ingredient. Chemical analysis of the rest of the inert ingredients will not be performed.

The protocol is being amended to change the specific gravity from 1.126 to 1.131. The change in specific gravity did not result in a change in the resulting ppm active ingredient at 0.25 oz/gallon for Dodecylbenzene Sulfonic Acid (LAS). The resulting ppm for Lactic Acid however will be amended from 648 ppm to 650 ppm. However, this change does not have an effect on the test substance use-solution dilution procedure. The use-solution acceptance criteria for Dodecylbenzene Sulfonic Acid (LAS) will remain unchanged but the use-solution acceptance criteria for Lactic Acid is being amended from 0.0583-0.0661% to 0.0585-0.0663%.

The protocol is being amended to attach MS111; *Antibiotic Susceptibility Tests* to the protocol.

Printed & Verified Initial & Date 11/6/18

Regulated Study Protocol Amendment

Study Title:	CW32A Supplemental Food Contact Sanitizing Efficacy
Study Number:	1800076
Amendment Number:	1800076-1A
Amendment Effective Date:	November 6, 2018

Scientific Basis for Amendment

The protocol was amended to clarify that the test substance use-solution chemical analysis was performed on CW32A batch P081781.

The protocol was amended to clarify why chemical analysis was performed on lactic acid when the common practice is to only perform chemical analysis on the active ingredients.

The protocol was amended to change the specific gravity from 1.126 to 1.131. The change in specific gravity did not result in a change in the resulting ppm active ingredient at 0.25 oz/gallon for Dodecylbenzene Sulfonic Acid (LAS) However, the resulting ppm for Lactic Acid did change from 648 ppm to 650 ppm. This change did not have an effect on the test substance use-solution dilution procedure or the use-solution acceptance criteria for Dodecylbenzene Sulfonic Acid (LAS). However, the use-solution acceptance criteria for Lactic Acid was changed from 0.0583-0.0661% to 0.0585-0.0663%. The change in the use-solution acceptance criteria for Lactic Acid has no effect on the study since the use-solution chemical analysis was determined to be within the new use-solution acceptance range.

The protocol was amended to attach MS111; *Antibiotic Susceptibility Tests* to the protocol since the method was previously listed in the Protocol Appendix but not actually attached to the protocol.

☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☒ This protocol amendment has been amended.
Refer to protocol amendment 1800076-2A for details.
Initial & Date 2/25/19

Study Director

11/6/18
Date

Printed & Verified Initial & Date 11/6/18

Form-Ver. 6016-09
Effective: 05/01/18
Form Page 1 of 1

Regulated Study Protocol Amendment

Study Title: CW32A Supplemental Food Contact Sanitizing Efficacy
Study Number: 1800076
Amendment Number: 1800076-2A
Amendment Effective Date: February 22, 2019

Description of Amendment

The protocol is being amended to include Lactic Acid as an active ingredient in addition to Dodecylbenzene Sulfonic Acid (LAS).

Scientific Basis for Amendment

The protocol was amended to include Lactic Acid as an active ingredient in addition to Dodecylbenzene Sulfonic Acid (LAS). Efficacy testing was conducted while waiting for a decision from the EPA on whether Lactic Acid would need to be declared an active ingredient or not. The EPA has now made the decision that Lactic Acid is an active ingredient. This recent decision does not have an affect on the efficacy testing since testing was previously performed with both actives at or below their lower limits.

☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☐ This protocol amendment has been amended.
Refer to protocol amendment _____ for details.
Initial & Date _____

Amanda Holen
Study Director

2/25/19
Date

Page 1 of 1

Printed & Verified Initial & Date AH 2/25/19